CHARACTERIZATION OF IN-HOUSE DEVELOPED ELECTROPORATOR

A Thesis submitted in partial fulfilment of the requirements for the degree of

Bachelor of Technology

In

Biomedical Engineering

By

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May, 2015

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CERTIFICATE

This is to confirm that the Thesis entitled "**Characterization of in-house developed electroporator"** by **Chadalavada Harshitha (111BM0479)** submitted to the National Institute of Technology, Rourkela for the honour of Bachelor of Technology in Bio-Medical building during the session 2011-2015 is a record of Bonafide research work did by her in the Department of Biotechnology and Medical Engineering under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University / Institute for the award of any Degree or Diploma.

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ABSTRACT

Electroporation is defined as an amplification in the electrical permeability and conductivity of the external cell membrane created by an externally applied electric field. At present substantial quantities of Electroporators are in the business sector, but they prove to be non-economical and cumbersome to use. In the current project, an in-house slide based, low cost electroporator was designed, fabricated and characterized. Briefly, a circular well of diameter 25 mm was made within a thick glass slide. It serves as a cuvette for the electroporator. The well was connected to electrodes made up of copper for supplying high voltage electrical impulse. The electronics of in-house electroporator comprised of five circuits.Firstly, a 555 Timer circuit to generate low amplitude pulses. Secondly, a 7805 regulator circuit to stabilize the voltage.Thirdly, a 12 Volt relay circuit to switch on/off the pulse generation. Fourth, the Microcontroller, 89C52 which can be programmed to control the pulses.Finally,a Transformer circuit with 1:100 turns ratio inorder to magnify pulse intensity necessary for the poration of the outer cell membrane. Thus, a wide range of variations in pulse amplitude and duration enables the Electroporator to be suitable for different cell types and molecules of different sizes. In the second phase, this Electroporator was characterized for precision in delivery of high voltage pulse for a short duration so that the cells would remain viable. The cells used were NIH3T3 Mouse embryonic fibroblast cells (NCCS,Pune) and the viability was estimated using MTT assay. The cells were first cultured.By keeping the pulse duration constant, the amplitude was varied. Next, keeping the the amplitude constant, the pulse duration was varied. In each case, MTT assay test was carried out for the cells which have experienced the electrical impulse. At each condition, the cells remained significantly viable. The cells were found to be most viable when subjected to 752V pulsating DC voltage with pulse duration 5 µs. The in-house electroporator was found to be effective in delivering the required voltage for required duration. Such a device would reduce the work process in research centers proper characterization for transfection.

Keywords: Electroporation, 555 Timer circuit,voltage,pulse width,cell culture,MTT assay

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CHAPTER-1

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INTRODUCTION

1.1 Transfection

Transfection is the process of introducing any nucleic acid molecules like DNA into cells. This technique is carried out by non-viral means in case of eukaryotic cells .

Depending on the sample to be injected into the cells, transfection can be categorised as:

1.1.1 SiRNA Transfection

This involves designing the necessary SiRNA against our gene and their subsequent delivery.

1.1.2 Plasmid DNA Transfection

Plasmid DNA is coupled with lipid reagents to facilitate effective delivery into the cell's nucleus. This process is necessary for expressing the gene of our interest.

1.2 Methods of Transfection

1.2.1 Chemical-based transfection

This involves Calcium phosphate transfection. This method has been in use since 1970's.The major reason being that the materials are not expensive and are easily available. Further, the technique is has an easy protocol and high efficiency. However, it is prone to vary due to its high sensitivity to temperature, pH, making it toxic to some cell types.

The principle involves mixing calcium chloride with DNA in a phosphate buffer solution to make a calcium phosphate–DNA co-precipitate. The generated co-precipitate is then dispersed onto the cultured cells. The calcium phosphate allows the binding of the DNA in the co-precipitate to the surface of the cell. The DNA then enters the cell by endocytosis.

1.2.2 Lipid-based transfection

The structure of cationic lipids consists of a head group that is positively charged and some hydrocarbon chains. The positively charged head group is responsible for the interaction between the lipid and the phosphate of the nucleic acid. This causes DNA condensation.

Liposomes with positive surface charge facilitate the interaction of the DNA and the outer cell membrane. This causes the transfection complex to be fused to the outer cell membrane, which is negatively charged. Later, the complex enters the cell through a process called endocytosis.

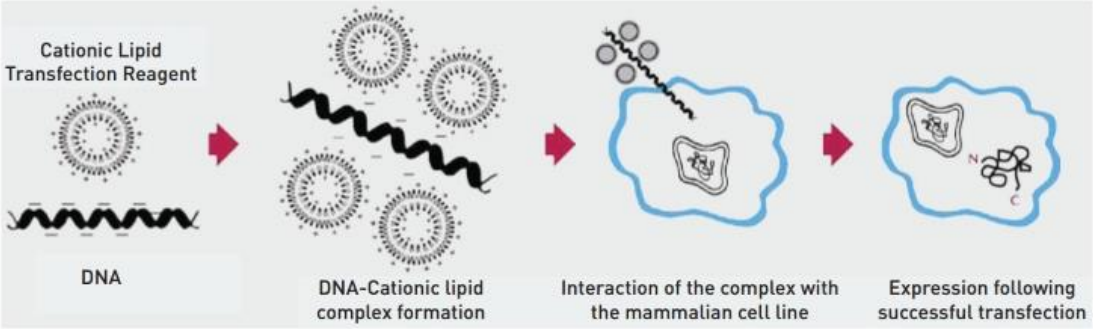

Figure 1.1: Mechanism of lipid-based transfection [1]

1.2.3 Physical-based transfection

This involves majorily the method of Electroporation. The cells of our interest are suspended in an electrically conductive solution, with an electrical circuit around the solution. The pulsating electrical signal of high voltage causes temporary pore formation. The electric potential across the outer cell membrane allows charged molecules like DNA to be transferred across the membrane through the pores [2].

The major advantage of this method is the formation of transient pores with the ability to transfect most of the cell types. It is an easy to perform, rapid and can be used to transfect multiple cells in a short span. One major disadvantage of electroporation is the cell death caused by high voltage.

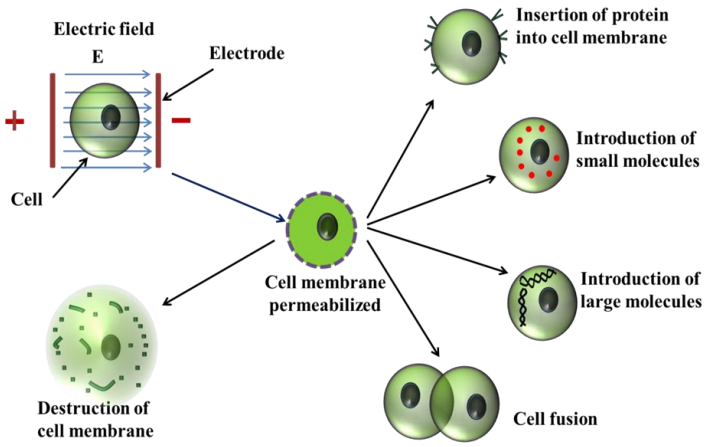

Figure 1.2: Mechanism of Cell Electroporation [3]

1.3 Objectives

Due to the above seen advantages of electroporation, this project comprises of accomplishment of the following :

1. Design and fabrication of an in-house electroporator having
   Output voltage = 500-1000 V
   Pulse duration= 5μs - 100ms
2. Characterization of the electroporator for it's precision in delivering electrical pulse without live cell damage using MTT assay.

CHAPTER- 2

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LITERATURE REVIEW

2.1 History

In 1994 low voltage electrical pulses were used as high voltage would damage the cells. Electroporation was first used in transfecting mouse embryos in the year 2001 by Osumi and Inoue [4]. Although since 18^{th} century many scientific results were exposed, the electroporation was not recognised as an increase of the permeability of the outer cell membrane until mid-20th century. Commercially, electroporation was tested in the 1960s as a bactericidal method for consumable goods. However, until early millenium, its use in medical industry was not considered. NanoKnife^{TM} developed by Angiodynamics, Inc. in 2008 was the first electroporation to be launched in the market .

2.2 Electroporator Mechanism

Electroporation follows a simple mechanism in which some cells or molecules are suspended in an electrically conductive solution, and an electrical current is passed through the mixture. An electrical pulse of duration in the order of millisecond, at a high voltage, is discharged through the mixture. This disturbs the phospholipid bilayer of the outer cell membrane and causes the formation of temporary transient pores.Unlike dielectric breakdown, during electroporation, the lipid molecules of the bilayer are not altered chemically. Instead, they simply shift their position. This causes a pore like opening, which provides a conductive path through the membrane into the cell.

Electroporation is a dynamic phenomenon. The extent of electroporation that depends on the local transmembrane potential at each distinct point on the outer cell membrane. For a given pulse duration and shape, a specific transmembrane potential threshold exists for the execution of the electroporation phenomenon. The areas on the outer cell membrane where the externally applied potential (E) is greater than or equal to the electric field magnitude threshold for electroporation (E_{th}) i.e. $E \geqq E_{th}$ are the areas where transient pore formation takes place [5].

The electrical potential generated across the outer cell membrane allow charged molecules like DNA to be driven across the membrane. An electroporator is highly expensive and sensitive device. It has applications ranging from introducing small substances into a cell, such as probes to drugs, or even DNA. This procedure is also very much useful in tumor therapy, gene therapy, and drug therapy.

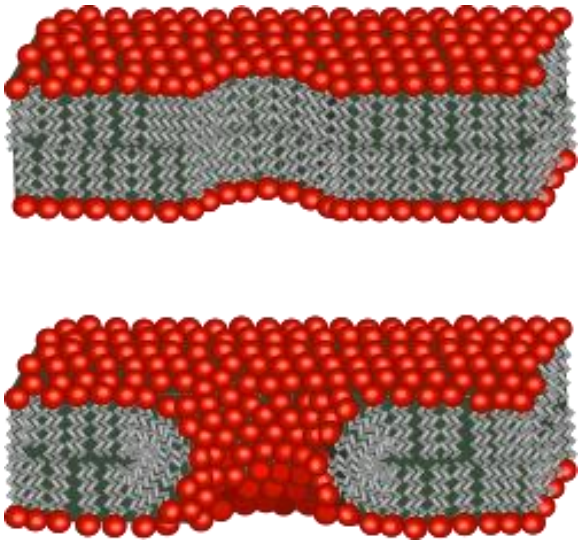


Figure 2.1: Arrangement of lipids before and during electroporation

2.3 Currently available Electroporators

2.3.1 Bio-Rad Gene Pulsar Xcell™ Electroporation System

Figure 2.2: Gene Pulsar Xcell™ Electroporation System [6]

Table 2.1: Gene Pulsar Xcell™ Electroporation System description

Specification	Description
Output	Waveform: Square or exponential Voltage: 10 to 3000 V
Capacitance	10 to 500 V, 25 to 3275 μF
Resistance (Parallel)	50 to 1000 Ω
Sample Resistance	20 Ω at 10 to 2500 V 600 Ω at 2500 to 3000 V
Square-wave timing	10 to 500 V: 0.05 ms in 0.05 ms increments

2.3.2 BTX Agile Pulse in vivo System (for vaccine development and gene therapy)

Figure 2.3: BTX Agile Pulse in vivo System [7]

Table 2.2: BTX Agile Pulse in vivo System Description

Specification	Description
User Interface	Touch Screen Display
Voltage Range	50 to 1000 volts
Pulse Width Range	0.050 to 10 ms
Pulse Interval	0.200 to 1000 ms (5 kHz to 1 Hz)
Data Export Dimensions	USB Flash Key 32 cm w x 20 cm h x 40 cm
Weight	25 pounds, 11.3 kg
Operating Temperature	10 to 40^{o}C

2.4 Electric field and cell types

The optimum field strength for succesful electroporation of different cell types is:

Table 2.3: Various cell types and their electroporation field strengths

Cell types	Field strength
Plant	3-12 KV/cm
Mammalian	0.25-3 KV/cm
Bacteria	3-24 KV/cm

The optimum voltage to be applied depend on three factors:

2.4.1. Cell Diameter

Larger cells require low voltages while smaller ones require high voltages

Table 2.4: Cell diameters and their electroporation voltages [8]

Cell diameter (in µm)	Cuvette 4mm (at room temperature)	Cuvette 4mm (at 4^{o} C)
50	100 V	200 V
40	130 V	250 V
30	180 V	360 V
20	250 V	500 V
15	350 V	700 V
10	500 V	1000 V

2.4.2 Cuvette gap size

The optimum voltage to be given for successful transfection can be calculated by multipying the field strength and cuvette gap size.Hence, for a fied strength of 1.5 KV/cm and cuvette gap size of 4mm, the voltage to be applied is 600 Volts.

2.4.3 Temperature

The voltage to be applied depends on the temperature also as shown in the above table.

2.5 Validation of electroporation

The success of the electroporation can be validated by two techniques:

2.5.1 Viability assay

It is an assay which is to determine the capacity of a cell to maintain its viabilty.It is used to quantify the live and dead cells.There are four categories according to which one can say whether the cell is viable or not. They are:

i. Mitochondrial activity assay (MTT)
ii. Membrane leakage assay (Trypan Blue)
iii. Genomic assay
iv. Functional assay [9]

MTT assay test is based on the quantitative measurement of extracellular reduction of yelow colored water soluble Tetrazolium dye to insoluble formazan crystals by metabolically active cells.

This reduction is mediated by mitochondrial enzyme lactate dehydrogenase. When dissolved in a appropriate solvent, these formazan crystals exhibit purple color, the intensity of which is proportional to the number of viable cells and can be measured spectrophotometrically at 570 nm.

2.5.2 Success of transfection

The success of the transfection can be determined in many ways. Two of the approaches include :

i. Plasmid DNA containing Green Fluoroscent Protein (GFP)
   Using a spectrophotometer one can measure the GFP fluoroscence intensity of the cells which determines the effiency of transfection. The GFP exhibits fluoroscence as it has a chromophore at the center of its helical structure.

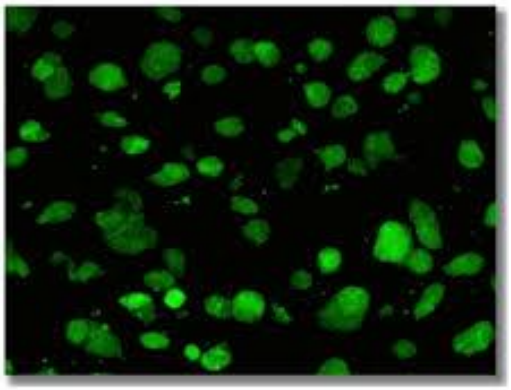


Figure 2.4: Green Fluroscent Protein

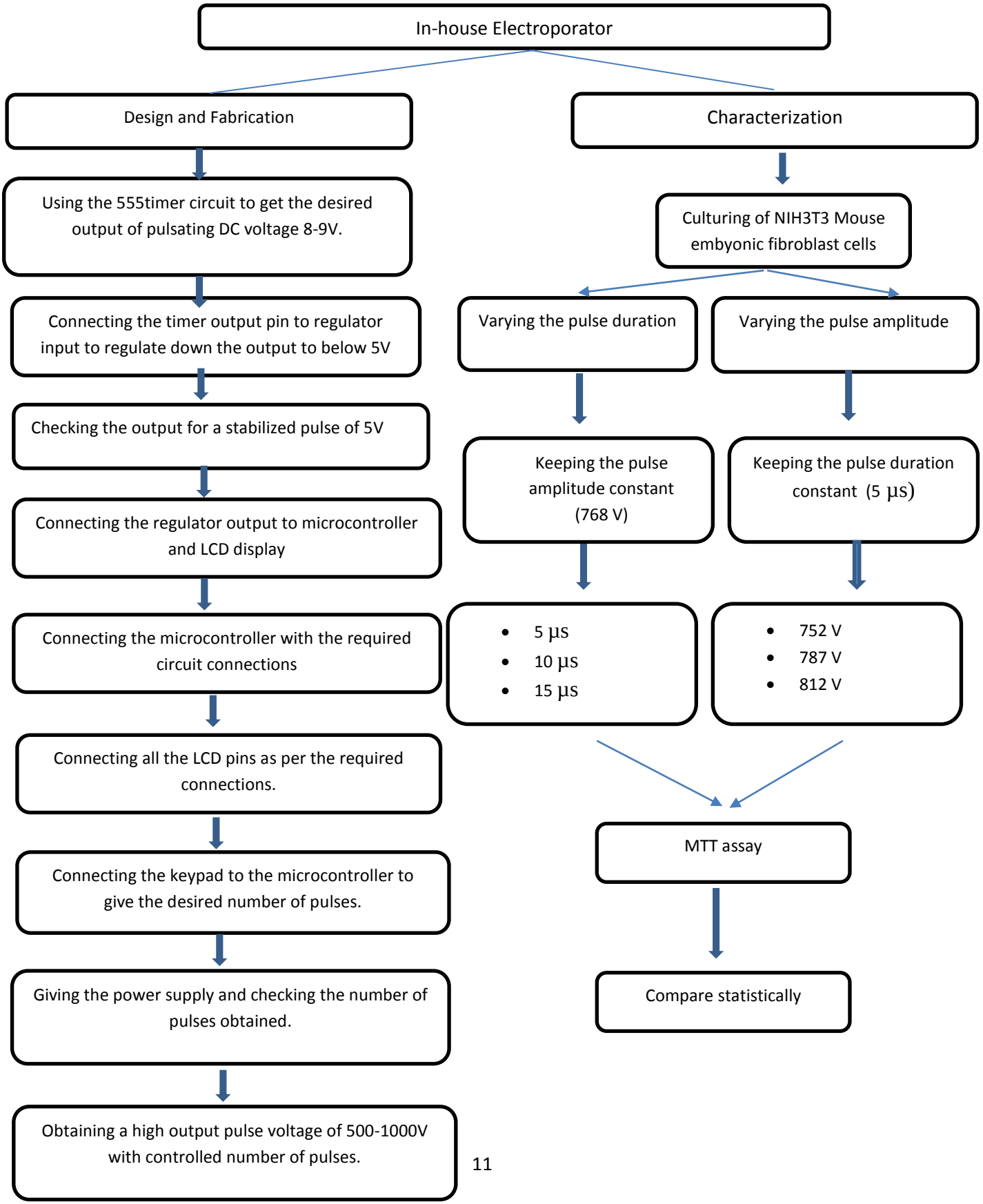
ii. Plasmid DNA containing Ampicillin-R gene

The success of transfecting the plasmid DNA containing ampicillin-R gene can be determined by zone inhibition test.If the transfection is successful, it implies that the cells now have an Amp-R gene. This means that no zone of inhibition should form.

Figure 2.5: Positive and negative results of zone of inhibition test

CHAPTER- 3

WORK FLOW



CHAPTER- 4

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MATERIALS AND METHODS

4.1 Design And Fabrication Of Electroporator

Components

1) 555 Timer
2) 7805 Regulator
3) Battery (9V)
4) Bread Board
5) Capacitors (10µF, 33pF)
6) Connecting Wires
7) Crystal (12 MHz)
8) Customized Glass Slide
9) Electrodes
10) Keypad
11) LCD Display
12) Micro Controller-89C52
13) Potentiometer
14) Relay(12V)
15) Resistors (1K, 10K, 100K)
16) Transformer

Instruments

17) Digital Storage Oscilloscope

18) Microcontroller Programming Kit

4.1.1 555 timer circuit

The 555 timer IC was used for the pulse generation and regulated the pulse duration of the Electroporator. A 9V continuous pulse waveform is obtained [10].

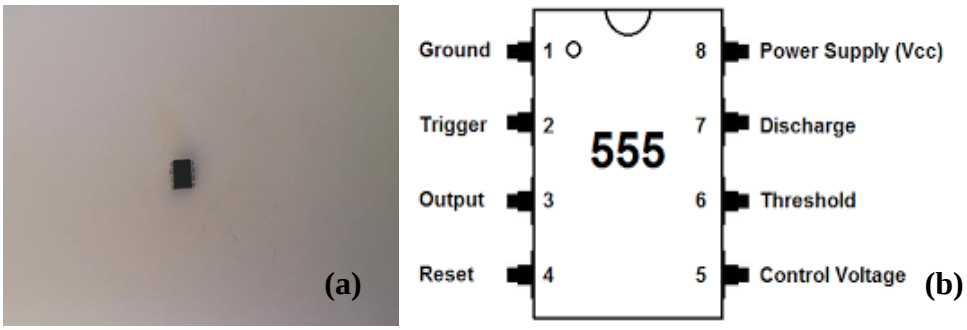


Figure 4.1: (a) 555 Timer Device (b) 555 Timer pin configuration

4.1.2 7805 Regulator

The LM7805 voltage regulator was used to regulate the DC voltage applied to a value less than 5V DC. The 7805 is easily available and not expensive.

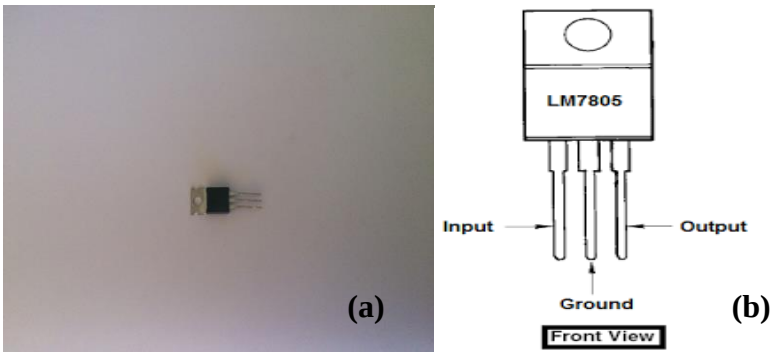


Figure 4.2: (a) 7805 Regulator Component (b) 7805 Regulator pin configuration

4.1.3 Battery

A 9V Li-ion battery (Hi-waote) was used as a power source to run the 555 Timer circuit and the LCD display [11].

Figure 4.3: 9V Battery component

4.1.4 Bread Board

It was used for arranging the circuit components on an insulating surface. It does not require any soldering of the components.Hence, it is reusable. The circuits can be made and even altered easily as it is solder free. This helps in the making of temporary circuits for experimental purpose [12].

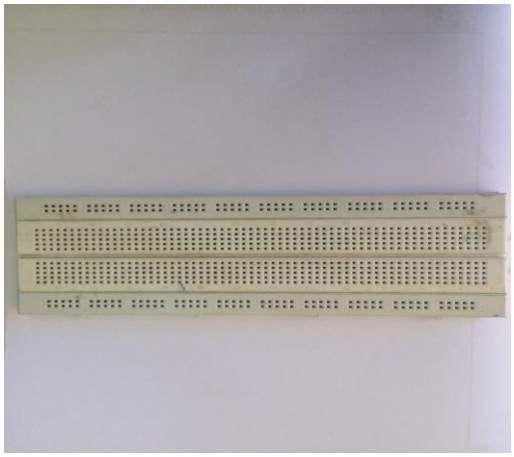


Figure 4.4: Bread Board

4.1.5 Capacitors

Capacitors (RAM Electronics Ltd., India) were used to generate the necessary time period of the pulsating DC output.With the help of Quartz crystal , it tunes the microcontroller circuit [13].

Figure 4.5: Capacitor Components

4.1.6 Connecting Wires

Connecting wires are used to join the components in an electrical circuit. The connections are temporary for bread board

Figure 4.6: Connecting wires

4.1.7 Crystal 12 MHz

A quartz crystal (SPK Electronics Co., Ltd., India) was used to monitor continuously the variations in frequency and provide an electrical signal with a constant frequency of 12 MHz that matches with the input of the microcontroller [14].

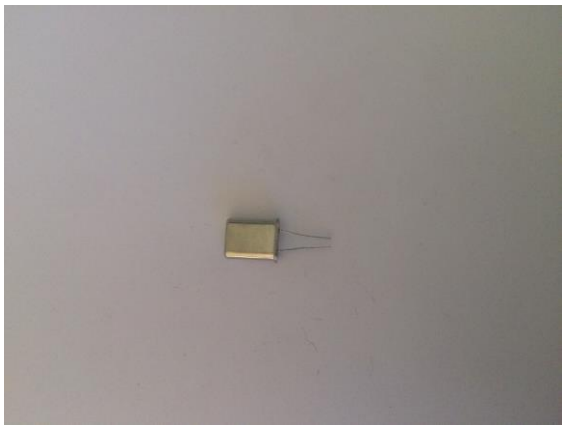


Figure 4.7: Crystal (12 MHz) Device

4.1.8 Customized Glass Slide

Customized glass slide was prepared by using the principle of extrusion. The the centre portion of a thick glass is removed in a circular shape of diameter 2.5 cm with help of round glass cutter. It is square shaped on its outer edges with dimensions of 10 cm x 10 cm and height of 1.4 cm.

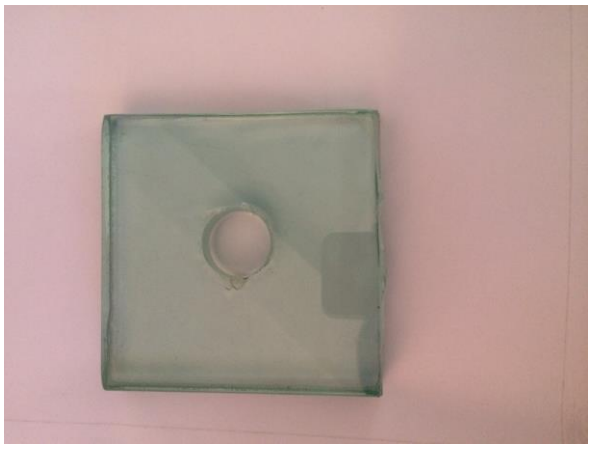


Figure 4.8: Customized glass slide

4.1.9 Electrodes

An electrode made up of copper was used to make contact with the cuvette of the electroporator [15].

Figure 4.9: Metallic Electrode

4.1.10 Keypad

A Keypad was used to give the required number of pulses to be generated.

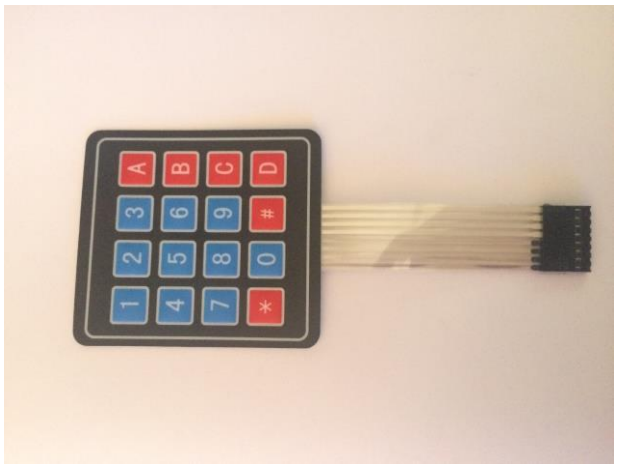


Figure 4.10: Keypad

4.1.11 LCD Display

Liquid-crystal Display (Formike Electronic Co., Ltd, India) was used to display the number of pulses given through the keypad. It runs with the power from the battery [16].

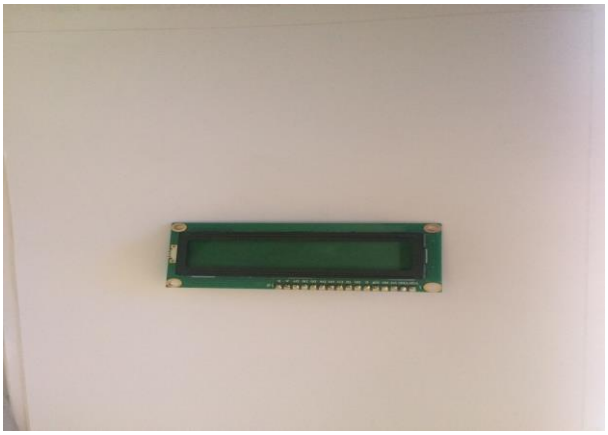


Figure 4.11: Liquid Crystal Display device

4.1.12 Microcontroller-89C52

Microcontroller (ATMEL Integrated Circuits, America) was used to control the number of pulses given by the output of 555 timer circuit. The 7805 regulator brings down the DC 9V to 5V to bring it to the Microcontroller range. Now, its output was given to a 12V relay which controls the required pulse number [17].

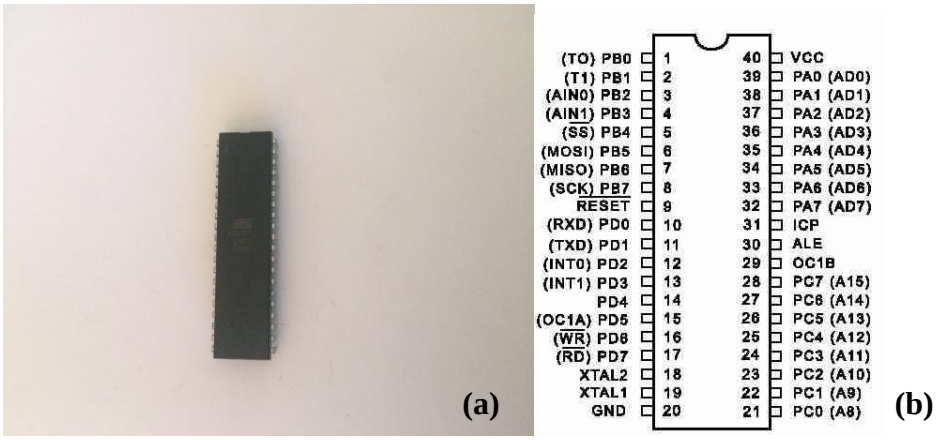


Figure 4.12: (a) Microcontroller Device (b) Microcontroller pin configuration

4.1.13 Potentiometers

Potentiometers (Bochen Chengdu guosheng technology co., Ltd., China) of 10KΩ was used to change the frequency of the output wave from 50 µs to 1000 ms. It was also used to change the peak to peak voltage of the output waveform from 9 to 800V [18].

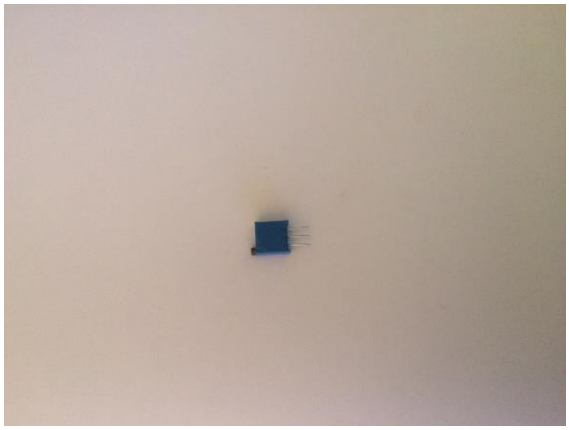


Figure 4.13: Potentiometer component

4.1.14 Relay

Relay of 12V (Zhejiang Zhongji Technology Co., Ltd, China) was used for controlling the pulses.When the relay is in the on mode,the pulses were generated and when the relay is in the off mode, pulses were stopped. The relay was controlled by the microcontroller [19].

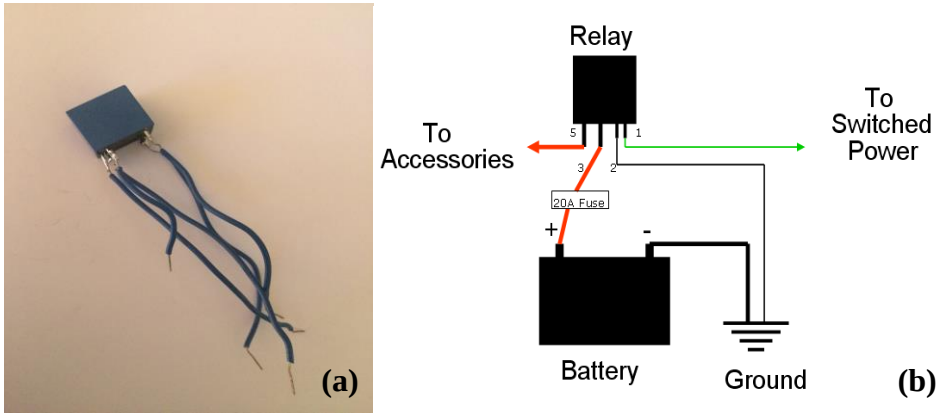


Figure 4.14: (a) Relay (12V) Device (b) Relay pin configuration

4.1.15 Resistors

Resistors (Cermet Resistronics Pvt. Ltd, India) of 1KΩ ,10KΩ and 100KΩ were used to generate the time constant for the pulse inorder to determine its frequency. [20].

Figure 4.15: Resistor Component

4.1.16 Transformer Circuit

The transformer (Metronix Pvt Ltd., India) was used to obtain a very high output voltage from a low input voltage. For this purpose, a transformer with turn ratio 80-100 was used keeping the frequency constant [21].

Figure 4.16: Transformer device

Instruments

4.1.17 Digital storage oscilloscope

A digital storage oscilloscope (Tektronix *India Private* Limited, America) was used to read and record the outputs from the 555 timer circuit, 7805 regulator circuit, 12V relay circuit, micro-controller circuit and transformer circuits. **[33]**.

Figure 4.17: Digital Storage Oscilloscope (DSO) instrument

4.1.18 Microcontroller programming kit

The programme kit (Pantech Prolabs India Pvt., Ltd.) was used for loading the programme into the Microcontroller 89C52. The programme was written) in C++ language and its HEX file for 89C52 was used to load the programme in the Microcontroller.

Figure 4.18: Microcontroller Programming kit

4.2 Circuit Diagram

The complete circuit diagram according to which the in-house electroporator has been designed is given below:

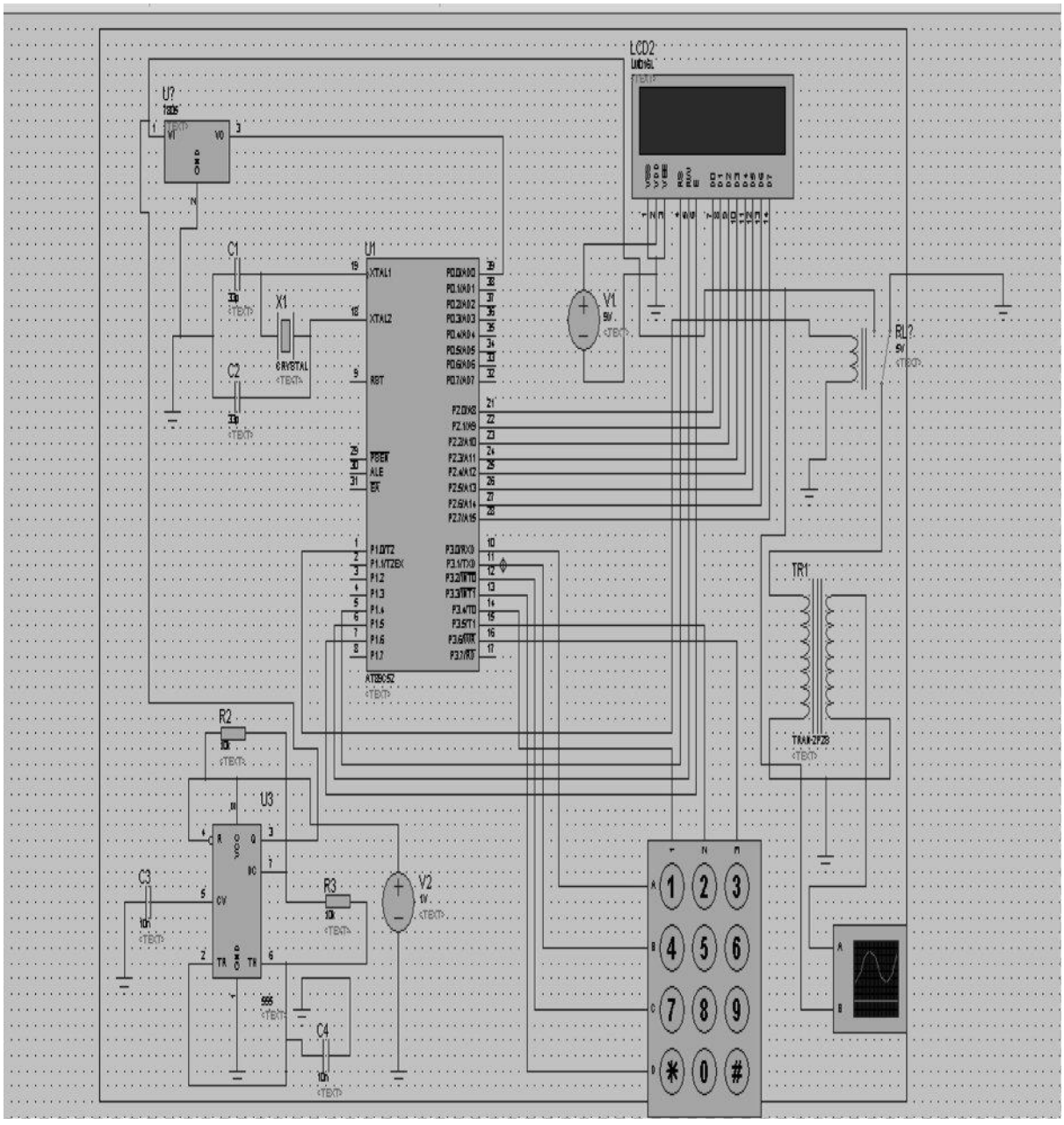


Figure 4.19: Circuit Diagram of the In-house Electroporation System

4.3 Culturing Of NIH3T3 Mouse Embyonic Fibroblast Cells

For cell viability test in response to the electrical pulse, NIH3T3 Mouse embyonic fibroblast cells were cultured using established protocol described elsewhere [23]. The cells were obtained from NCCS, Pune.

4.3.1 Preparation of complete media

Briefly, 4ml FBS was taken in a falcon tube. 400 µl of antibiotic (Ampicillin) was added. Now, 200 µl of antifungal (Amphotericin) was added. The solution was made to 40 ml by adding Low Glucose DMEM. The falcon tube was shook 8 to 12 times until the foam was formed.

4.3.2 Cell culturing

Briefly, 15 ml of complete media was transferred to the T-75 flask. The retrieved cells were transferred to the T-75 culture flask. The cells were mixed properly with the complete media. The T-75 culture flask was placed into the CO_2 incubato. The cells were observed after 24 hours.

4.3.3 Subculturing

Briefly, the culture media and Trypsin-EDTA were pre-warmed to 37^o C in water-bath. The cells were removed from the incubator and the media was aspirated. The flask was wash with PBS and aspirated. Appropriate amount of Trypsin-EDTA was added to sufficiently cover the surface area. Cells were rinsed in Trypsin-EDTA by gently swirling the flask. Most of the trypsin was aspirated , leaving behind just enough to form a thin monolayer on the flask surface. The cells were then incubated at 37^{o}C. After some time, the cells were removed from the incubator and were observed under the microscope. When the cells appeared sufficently rounded, the flask was put to detach the cells completely. Appropriate amount of fresh complete media was added and mixed well. The cells were transferred to a new flask. The cells were observed under the microscope and were returned back to the incubator.

4.4 Application of current through Electroporator

Inorder to characterize the in house developed electroporator, the cultured NIH3T3 Mouse embyonic fibroblast cells were divided into six sets.Three sets were subjected to varied pulse ampllitudes at constant pulse duration and another three sets ubjected to varied pulse duration at constant pulse amplitudes.The viability of the six sets of cells after electroporation was tested through MTT assay [24].

Table 4.1: Characterization of Electroporator

Cell Set Number	Pulse Amplitude applied (Volts)	Pulse duration applied (microsecond)
Keeping Amplitude constant		
1	768	5
2	768	10
3	768	15
Keeping Pulse duration constant		
4	752	5
5	787	5
6	812	5

4.5 Viability Assay

For cell viability, MTT assay was performed using established protocol described elsewhere [25].

4.5.1 Preparation of MTT reagent

Aseptically add 6 ml of cell based assay buffer in one MTT vial and completely dissolve the powder. The powder disolves slowly in the buffer. Vigorous vortexing is needed to dissolve the powder completely. Concentration of the resulting solution is 5 mg/ml. MTT solution should appear bright yellow in color.

4.5.2 Preparation of cells

The subcultured cells (3 sets) were subjected to three different pulse amplitude at constant pulse duration and another 3 sets were subjected to three different pulse durations at constant pulse amplitude.Now, the MTT assay test was conducted.

4.5.3 Protocol

Briefly, 100 µl of each diluted set of cells in 96-well microtiter plate was taken. Medium control was added to the cells. Cells were incubated under appropriate conditions depending on the cell line under study. 10 µl of MTT was added to each well, including controls. The plate was wrapped with aluminium foil to avoid exposure to light. The plate was returned to the incubator for 2-4 hours. Cells were observed at periodic intervals under an inverted micoscope for presence of intracellular needle-shaped, dark purple colored precipitate. When the purple precipitate was clearly visible, 100 µl of solubilization solution was added to the wells. Gentle stirring on a gyratory shaker was done to enhance dissolution of crystals. Absorbance on a UV spectrophotometer at 570 nm was read and plotted.

CHAPTER- 5

RESULTS AND DISCUSSION

5.1 Complete set-up of the In-house Electroporator

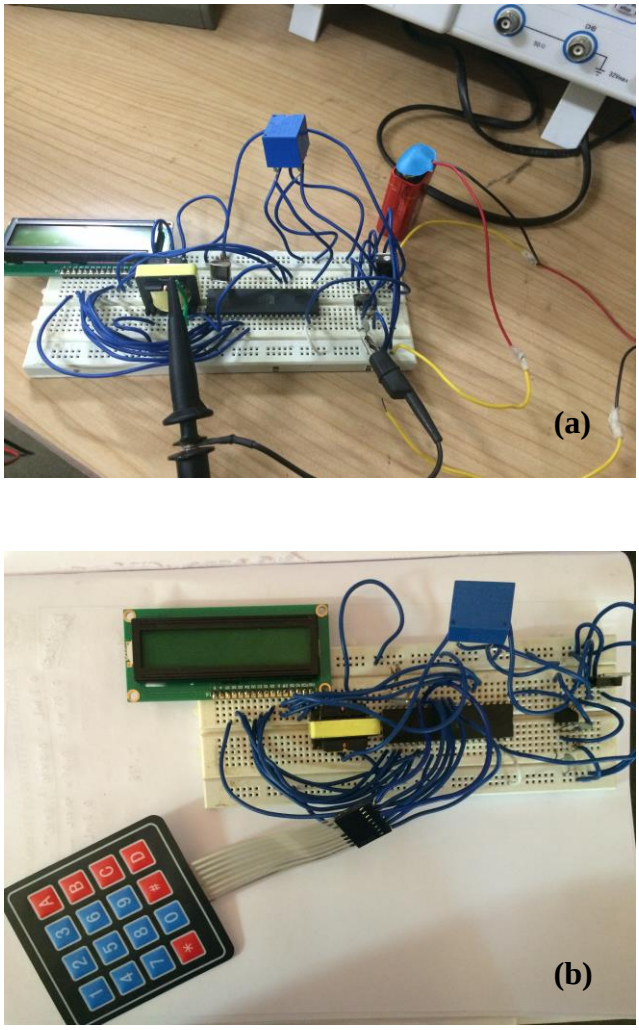


Figure 5.1: (a)Complete Set-up of the In-house Electroporator (Side view) (b) Top View

5.2 Experimental Results of Electroporator

5.2.1 555 Timer circuit output

The 555 Timer circuit was connected according to the pin configuration and circuit diagram its output waveform was observed. The result was a pulsating DC signal of 8-9 V range.

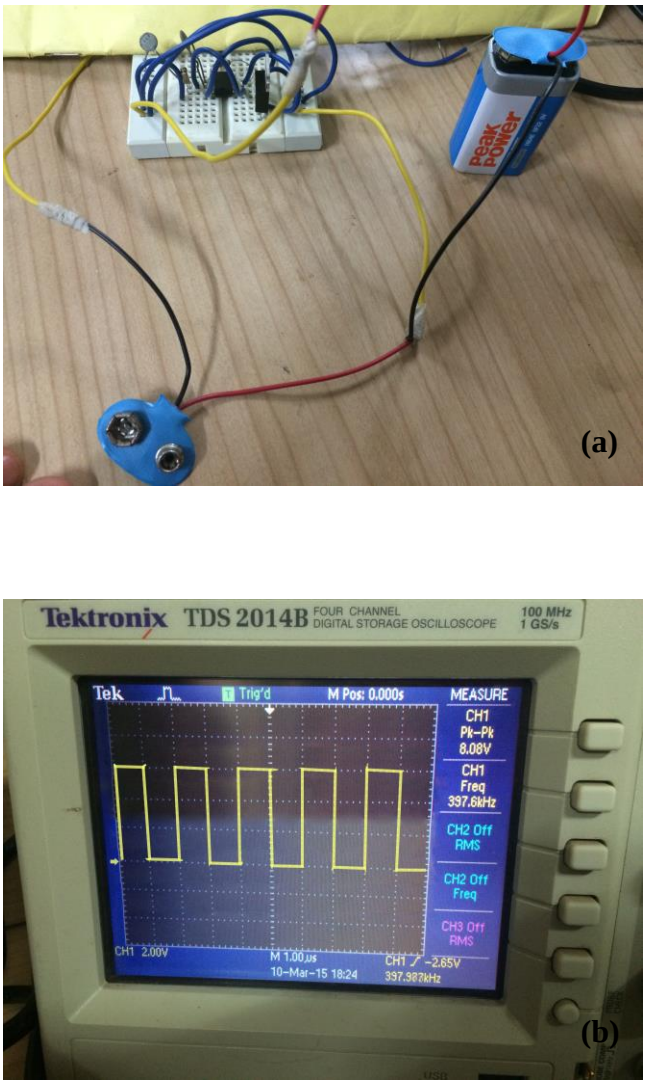


Figure 5.2: (a)555 Timer Experimental Set-up (b) 555 Timer Experimental Output

5.2.2 Regulator circuit output

The 555 timer circuit output was connected to the 7805 regulator input . The result was a down regulated output waveform of range 4-5 V.

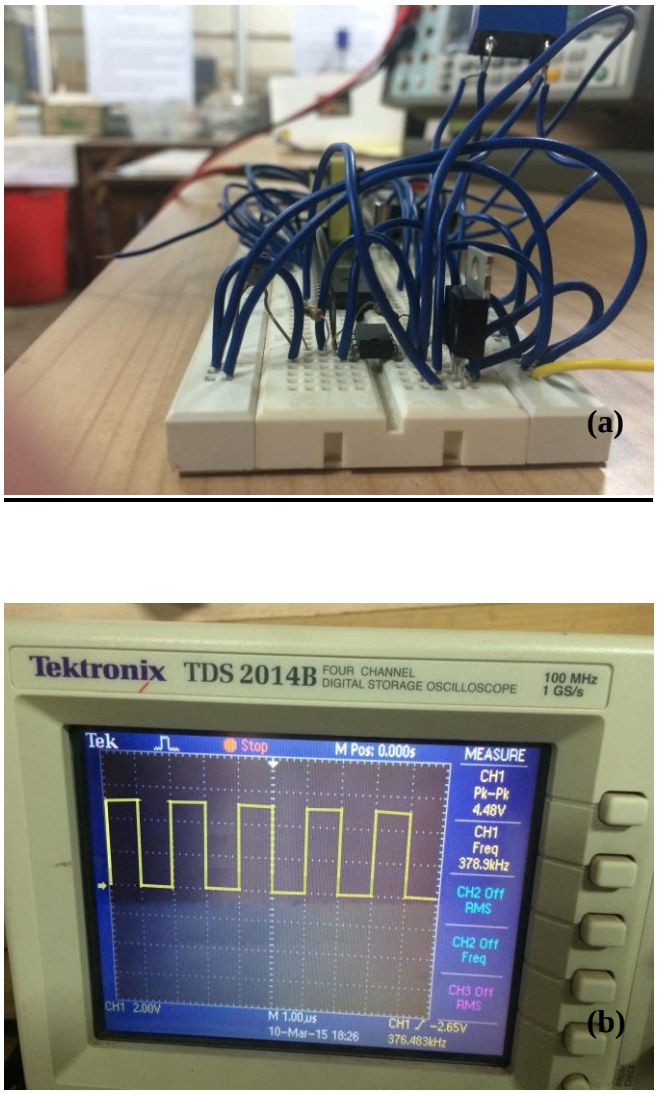
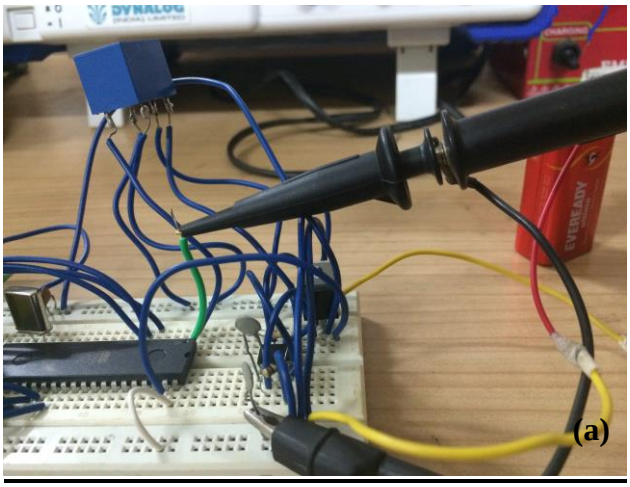


Figure 5.3: (a) 7805 Regulator Experimental Set-up (b)7805 Regulator Experimental Output

5.2.3 Microcontroller relay circuit output

The output of the regulator circuit was given to the relay circuit. The number of pulses were controlled and 14 pulses through programming were given and achieved.



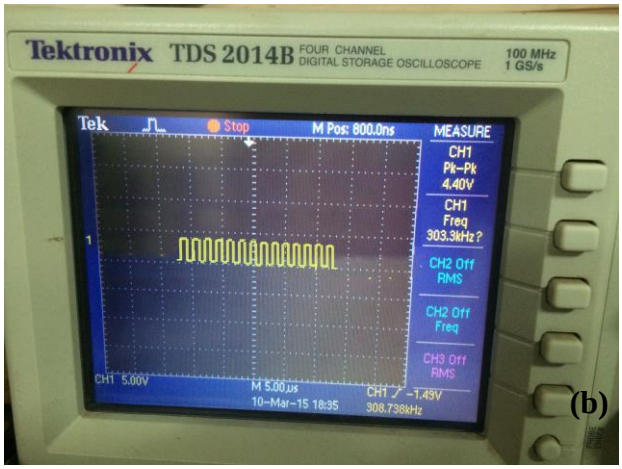
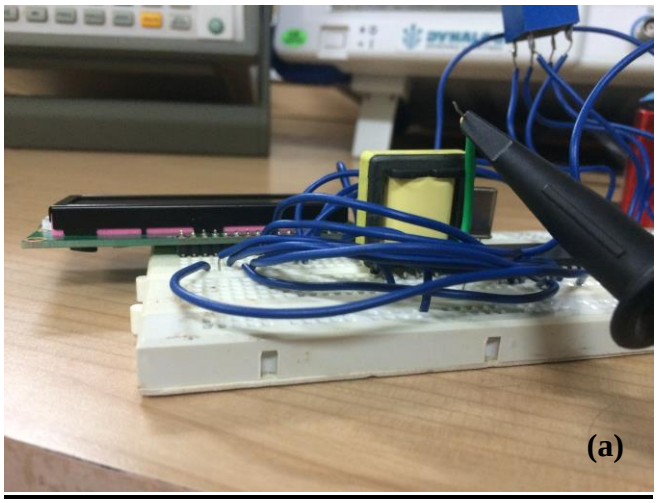


Figure 5.4: (a) Microcontroller Experimental Set-up (b) Microcontroller Experimental Output

5.2.4 Microcontroller transformer circuit output

The relay output pulse was given to the input of the transformer circuit to control the number of pulses and gave 3 pulses through programming.



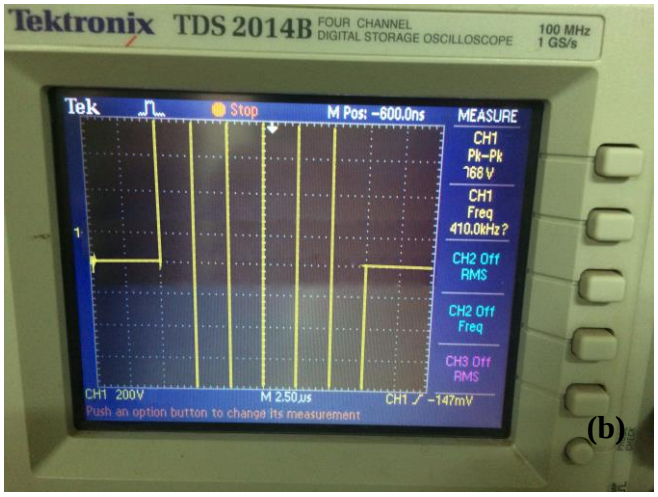


Figure 5.5: (a) Transformer Experimental Set-up (b) Transformer Experimental Output

5.3 Experimental results of MTT assay

The optical density (OD) values obtained for each set of cells through MTT assay is described below:

Table 5.1: Cell viability result after being subjected to elctrical pulse

Cell Set Number	Pulse Amplitude applied (Volts)	Pulse duration applied (microsecond)	Absorbance (OD)
Keeping Amplitude constant			
1	768	5	0.78
2	768	10	0.75
3	768	15	0.65
Keeping Pulse duration constant			
4	752	5	0.8
5	787	5	0.7
6	812	5	0.6

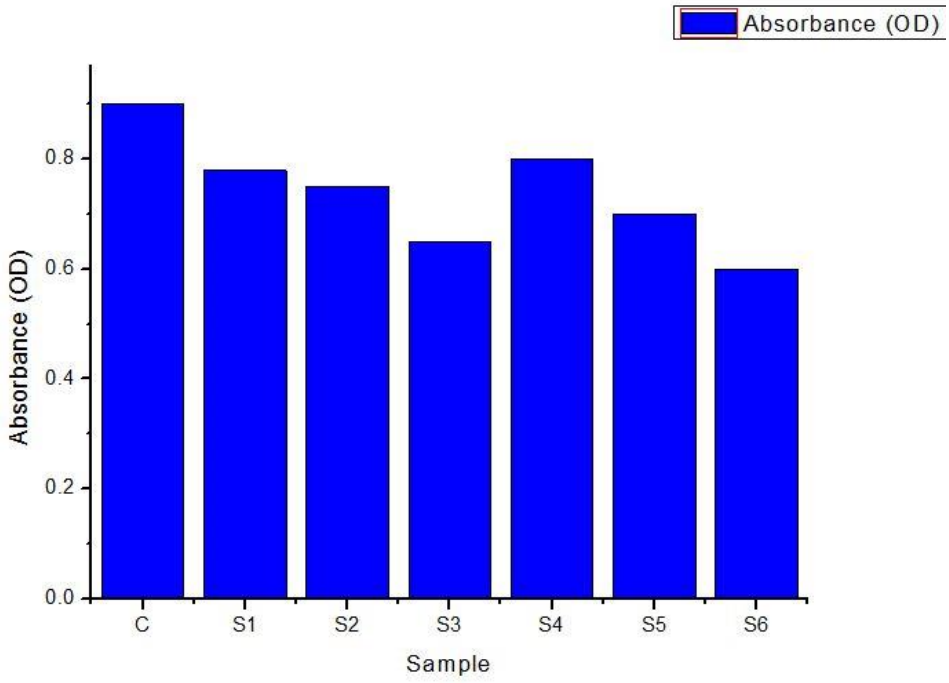


Figure 5.6: Graphical representation of MTT assay

CHAPTER- 6

CONCLUSION

Hence an in-house low cost electroporator was designed and fabricated.The electroporator was characterized by validating through MTT assay. The cells were found out to be the most viable when subjected to 752V DC pulsating voltage with pulse duration 5 µs.

CHAPTER- 7

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